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ORIGINAL ARTICLE

Characterization and complete genome sequence analysis of two *Myoviral* bacteriophages infecting clinical carbapenem-resistant *Acinetobacter baumannii* isolates

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Acinetobacter baumannii, antimicrobial alternatives, bacteriophage, biocontrol, carbapenem, Myoviridae.

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Abstract

Aims: The aim of this study was to characterize phenotypical properties, to analyse whole genomes of novel *Acinetobacter baumannii* phages infecting carbapenem-resistant *Ac. baumannii* (CRAB) and to evaluate their potential as antimicrobial alternatives to control *Ac. baumannii* in clinical settings.
Methods and Results: The *Ac. baumannii* phages, Bϕ-R1215 and Bϕ-R2315, were isolated from sewage samples. These phages were characterized by transmission electron microscopy, host spectrum, the thermal/pH stability test, the bacterial lysis assay and the whole genome analysis. Both phages lysed 21 of 45 CRAB hosts, and showed high stability at various pH (pH 4–10) and temperature (25–60°C), and were strongly active against host bacteria *in vitro*. The genomes of Bϕ-R1215 and Bϕ-R2315 are linear double-strands of DNA with 44·866 and 44·846 bp respectively. These two genomes revealed high similarity at the DNA level, but the organization and direction of open reading frames were different.
Conclusions: The *Ac. baumannii* phages, Bϕ-R1215 and Bϕ-R2315, are novel lytic phages lysing CRAB strains which were isolated from respiratory samples of patients.
Significance and Impact of the Study: *In vitro* and *in silico* data showed that these novel *Ac. baumannii* phages, Bϕ-R1215 and Bϕ-R2315, have potential as antimicrobial alternatives to control CRAB in healthcare settings.

Introduction

Acinetobacter baumannii is an opportunistic pathogen that has caused severe hospital-associated infections worldwide (Dijkshoorn *et al.* 2007; Munoz-Price and Weinstein 2008). In recent decades, the wide use of broad-spectrum antibiotics has caused increased rates of resistance in *Ac. baumannii* to most commercially available antibiotics (Dijkshoorn *et al.* 2007; Magiorakos *et al.* 2012). Carbapenem antibiotics are the last resort to treat Gram-negative bacterial infections; however, *Ac. baumannii* is even resistant to carbapenems including imipenem and meropenem (Nordmann and Poirel 2002). These resistances make treatment difficult in intensive care units and have caused great medical concerns worldwide (Nordmann and Poirel 2002; Higgins *et al.* 2010; Chung *et al.* 2014); therefore, more effective treatment plans are in demand. As a new alternative strategy to control antibiotic-resistant bacterial infections, bacteriophage therapy has recently been applied (Kutateladze and Adamia 2010; Burrowes *et al.* 2011).

After the discovery of phages by Frederick Twort in 1915 and Felix d'Herelle in 1917, phage therapy had been studied and used to treat bacterial infections all over the world, particularly in the United States and in the Eastern European countries of Georgia and Russia (Alisky *et al.*

1998; Abedon *et al.* 2011). The 1940s marked the start of an antibiotics era with extensive development and use of antibiotics; this movement detracted interest from phage therapy in the Western world (Sulakvelidze *et al.* 2001). However, recently, with the increased prevalence of serious problems with antibiotic-resistant bacteria, there is a renewed interest in phages as an alternative to antibiotics for the control of bacterial infections in animals and humans (Bruttin and Brussow 2005; O'Flaherty *et al.* 2009; Abedon *et al.* 2011). In 2006, phage agents were approved as additives against food-borne pathogens in the United States (Mahony *et al.* 2011). Therefore, we expect that bacteriophages can be developed into excellent biocontrol agents in the clinical setting as well.

Till date, complete genome sequences and characterization of approx. 20 *Ac. baumannii* phages have been reported in the NCBI database (http://www.ncbi.nlm.nih.gov/pubmed, Accessed: 10 October. 2015). Most of these phages belong to one of the three families in the order Caudovirales—Myoviridae, Podoviridae or Siphoviridae (Peng *et al.* 2014). These data, in addition to physiological data, can be used to extend our understanding about the features of *Ac. baumannii* phages. However, only few physiological characterizations of these phages have been reported. For the successful applications of *Ac. baumannii* phages as alternative natural antimicrobials, discovery of novel phages and more detailed studies on physiological and genetic aspects is essential.

In this study, two novel Myoviridae lytic phages (phage Bϕ-R1215 and Bϕ-R2315) multidrug-resistant organisms, specifically carbapenem-resistant *Ac. baumannii* (CRAB) strains were isolated and physiologically characterized. Also, these phages compared with other *Ac. baumannii* phages by whole genome sequence analysis. This information will aid in the development of phage-based treatments for the control of *Ac. baumannii*-associated clinical infections in hospitals.

Materials and methods

Bacterial strains and characterization

A total of 45 CRAB strains were isolated from clinical samples of patients from 2011 to 2014. These strains were identified using the VITEK 32GN system (bioMérieux, Marcy l'Etoile, France) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (VITEK MS; bioMérieux). An antimicrobial susceptibility test was performed using the disk diffusion method and the VITEK N132 system (bioMérieux), and the data were analysed as per the Clinical and Laboratory Standards Institute guidelines (Wikler and Clinical and Laboratory Standards, I. 2010). Specifically, CRAB YMC11/12/R1215 and YMC11/12/R2315 isolates, which were isolated from respiratory samples, were used as host bacteria for the phages in this study.

Isolation and purification of phages

The two *Ac. baumannii* phages were isolated from a sewage sample and were purified by the double-layer agar plate method (Kropinski *et al.* 2009). Briefly, sodium chloride (1 mol l^{-1}, Merck) and polyethylene glycol (PEG) 8000 (at a final concentration of 10%) (Sigma, St. Louis, MO) were added to the sewage samples and incubated at 4°C for 24 h. After centrifugation (8000 *g* for 10 min at 4°C) (Himac CP100WX; Hitachi, Tokyo, Japan), the supernatant was filtered using a 0·22 μm membrane filter (Millipore Corporation, Bedford, MA). Phages were pelleted by centrifugation at 12 000 *g* for 1 h at 4°C, and resuspended in sterilized sodium chloride-magnesium sulphate (SM) buffer (100 mmol l^{-1} NaCl, 8 mmol l^{-1} $MgSO_4$, 2% gelatin, 50 mmol l^{-1} Tris-HCl, pH 7·5).

Spot tests were performed by spotting 10 μl of phage solution onto bacteria lawns on a Luria–Bertoni (LB, Difco) agar plate. After overnight incubation, clear zone formations were checked.

To purify phages, the double-layer agar plate method was used to collect single-plaque isolations, which were harvested and suspended in 1 ml of SM buffer. These steps were carried out at least three times in order to select the phages. To concentrate the phages, host strains (OD_{600} = 0·5) and 10 μl of purified phage solution were added to 4 ml of LB medium and incubated at 37°C for 12 h with shaking. Culture samples were centrifuged at 12 000 *g* at 4°C for 10 min and filtered to remove cell debris. Then, PEG 8000 was added to a final concentration of 10% and the phage solution was centrifuged at 12 000 *g* at 4°C for 10 min. Subsequently, the solution was incubated at 4°C for 12 h. The samples were then resuspended in 1 ml of SM buffer and the concentrated phage solution was stored at 4°C. Plaque assays were performed to determine the number of plaque-forming units (PFUs) in the phage solutions using the double-layer method.

Host range of phages

A host range test was performed with *Ac. baumannii* phages on strains of bacteria that were isolated from patients at the hospital: 45 CRAB, five carbapenem-susceptible *Ac. baumannii*, five *Pseudomonas aeruginosa*, five *Escherichia coli*, five *Bacillus subtilis* and five *Staphylococcus aureus*.

The sensitivity of the phages to the collected clinical strains was evaluated by the spot test and efficiency of plating (EOP) using the double-layer agar plate method. The EOP is presented as a ratio of the phage titre on the host strain to the titre on other tested strains (Frampton *et al.* 2014).

Transmission electron microscopy

Purified phages (approx. 10^{10} PFU ml^{-1}) were laid on a carbon-coated copper grid and stained with 2% (w/v) uranyl acetate for 15 s. Prepared phage samples were viewed using a transmission electron microscope (JEOL JEM-1011, Tokyo, Japan) at 80 kV.

pH stability and thermal stability test

pH stability of the phages (10^6 PFU ml^{-1}) was evaluated at pH values ranging from pH 4 to 10 for 30 days at 4°C. The phage survival was measured at day 1, 3, 5 and 30. The titre of all samples was assayed by using the double-layer agar plate method. The data were compared with those of the control samples (10^6 PFU ml^{-1}, pH 7·5). To evaluate the thermostability, the phage suspensions (10^6 PFU ml^{-1}) were incubated at 25, 40, 50, 60 or 70°C for 3, 6 or 9 h (15 time/temperature combinations). These results were compared with the control titre (at 4°C, pH 7·5 for each time point), and all results were expressed as the percentage of phage titre (PFU ml^{-1}).

Host cell lytic activity test

Acinetobacter baumannii YMC11/12/R1215 and YMC11/12/R2315 culture strains (OD_{600} = 0·2) were infected with phages at different multiplicity of infections (MOIs, MOI = 0·1, 1, 10) at 37°C with continuous shaking. Each sample was analysed for host cell lytic activity at 1 h intervals (up to 6 h) by assessing changes in OD_{600} (spectrophotometer: Shimadzu-1601, Tokyo, Japan). An uninfected culture was used as a control and this experiment was conducted in triplicate.

DNA preparation and sequencing analysis of phages

Genomic DNA of phage Bϕ-R1215 and Bϕ-R2315 was extracted using the phenol extraction method as previously described (Wilcox *et al.* 1996) and was sequenced using a 454 GS Junior Genome analyser (454 Life Sciences/Roche, Branford, CT). The sequencing reads were assembled using Roche GS Assembler ver. 2.6 (Roche) and CLC Genomics Workbench 6.5 (CLCbio USA, Cambridge, MA). The open reading frame (ORF) prediction was carried out with NCBI ORF finder (http://www.ncbi.nlm.nih.gov/projects/gorf/) and GenMark.hmm software (Besemer *et al.* 2001). Overall genome sequences of the phages were compared with other phages using the Blast2seq tool, and the similarity of the predicted ORFs was determined using psi-blast (http://www.ebi.ac.uk/Tools/sss/fasta/). Putative protein functions of the ORFs were assessed using blastp in the NCBI database and InterProScan 5 on the EMBL-EBI Website (http://www.ebi.ac.uk/Tools/pfa/iprscan5/). Putative tRNA-encoding genes were analysed using tRNAscan-SE 1.21 (http://lowelab.ucsc.edu/tRNAscan-SE/). A map of the phage genome was presented using DNAPlotter (Carver *et al.* 2009), and genome organization with other phages was compared using mauve software ver. 2.3.1 (http://darlinglab.org/mauve/mauve.html).

Nucleotide sequence accession number

The complete genome sequences of *Ac. baumannii* phage Bϕ-R1215 and Bϕ-R2315 are accessible in the GenBank database under accession numbers KP861231 and KP861229 respectively.

Results

Identification of bacteria

A total of 45 CRAB strains were isolated from patients at a tertiary hospital in South Korea (Table 1). All strains were used for the host range determination of *Ac. baumannii* phages Bϕ-R1215 and Bϕ-R2315. Among them, *Ac. baumannii* YMC11/12/R1215 and *Ac. baumannii* YMC11/12/R2315 isolates which showed resistant to all antibiotics, except colistin (Table S1) were used as host bacteria for the preparation of the phages in this study.

Morphology and host range test

Transmission electron microscopy revealed that Bϕ-R1215 and Bϕ-R2315 had a head length of approx. 52 and 53 nm in diameter and contractile tails of approx. 79 and 77 nm in length respectively. Therefore, it was determined that both phages belong to the family Myoviridae (Fig. 1). Both *Ac. baumannii* phages formed clear plaques on 21 of 45 CRAB strains, 1 of 5 carbapenem-susceptible *Ac. baumannii* strains, and 1 of 5 *Staph. aureus* strains. However, they did not lyse clinical strains of *Ps. aeruginosa*, *E. coli* or *B. subtilis* (Table 1).

pH and thermal stability test

To investigate the stability of the phages on various environmental conditions, we observed the survival rate of

Table 1 Bacteria strains used in this study and their sensitivity to *Acinetobacter baumannii* phage Bϕ-R1215 and Bϕ-R2315

Species and strains		Sensitivity* to phage Bϕ-R1215	EOP†	Sensitivity to phage Bϕ-R2315	EOP	Species and strains		Sensitivity to phage Bϕ-R1215	EOP	Sensitivity to phage Bϕ-R2315	EOP
Carbapenem-resistant	YMC11/12/R1215	+	1·0	+	0·6	Carbapenem-resistant	YMC13/01/R317	−		−	
Ac. baumannii	YMC11/12/R2315	+	0·9	+	1·0	*Ac. baumannii*	YMC13/01/C62	−		−	
	YMC11/10/R3285	+	0·5	+	0·6		YMC13/01/U249	−		−	
	YMC11/10/R3128	+	0·7	+	0·6		YMC13/01/R280	−		−	
	YMC11/10/R3278	+	0·5	+	0·8		YMC13/01/R224	−		−	
	YMC11/11/R430	+	0·6	+	0·8		YMC13/01/R656	−		−	
	YMC11/11/R1255	+	0·5	+	0·6		YMC13/01/P187	−		−	
	YMC11/11/R2096	+	0·6	+	0·7		YMC13/01/R1400	−		−	
	YMC11/12/R270	+	0·8	+	0·7		YMC13/01/R1224	−		−	
	YMC11/03/R1520	+	0·6	+	0·6		YMC13/01/R1919	−		−	
	YMC11/11/R1255	+	0·7	+	0·8	Carbapenem-sensitive	YMC11/01/B6871	−		−	
	YMC11/11/R3252	+	0·7	+	0·9	*A. baumannii*	YMC11/02/B6287	−		−	
	YMC11/11/R3271	+	0·6	+	0·9		YMC11/02/B6674	−		−	
	YMC11/11/R3177	−		−			YMC11/02/B6127	+	0·6	+	0·8
	YMC11/11/P867	−		−			YMC11/02/B7203	−		−	
	YMC11/12/R23	−		−		*Pseudomonas aeruginosa*	YMC12/01/R21	−		−	
	YMC11/12/R33	+	0·6	+	0·8		YMC12/01/R1679	−		−	
	YMC11/12/R77	−		−			YMC11/01/R263	−		−	
	YMC11/12/T21	+	0·7	+	0·8		YMC11/02/R656	−		−	
	YMC11/12/U39	+	0·5	+	0·8		YMC11/03/P287	−		−	
	YMC11/11/T617	−		−		*Escherichia coli*	YMC11/12/B724	−		−	
	YMC11/12/R23	−		−			YMC11/12/B540	−		−	
	YMC11/12/R104	−		−			YMC11/12/B372	−		−	
	YMC11/12/R125	−		−			YMC11/12/B442	−		−	
	YMC11/12/R135	+	0·8	+	0·8		YMC11/12/B142	−		−	
	YMC11/12/R270	+	0·8	+	0·8	*Bacillus subtilis*	YMC14/03/R965	−		−	
	YMC11/10/P356	+	0·6	+	0·7		YMC14/01/B2619	−		−	
	YMC11/12/P447	+	0·7	+	0·6		YMC14/01/B3680	−		−	
	YMC11/12/R1828	+	0·9	+	0·9		YMC14/01/B5073	−		−	
	YMC13/01/R129	−		−			YMC11/01/B4235	−		−	
	YMC13/01/R2058	−		−		Staphylococcus aureus	YMC11/04/C397	−		−	
	YMC13/01/R1238	−		−			YMC11/04/P382	−		−	
	YMC13/01/R3197	−		−			YMC11/04/P383	−		−	
	YMC13/03/R12096	−		−			YMC11/04/P387	+	0·1	+	0·1
	YMC13/04/B720	−		−			YMC11/04/P367	−		−	

*Phage activity against collected bacteria: +, clear plaque; −, no plaque.

†The efficiency of plating (EOP) was calculated as the titre (PFU ml^{-1}) on the test bacteria strain divided by titre (PFU ml^{-1}) on host bacteria strain.

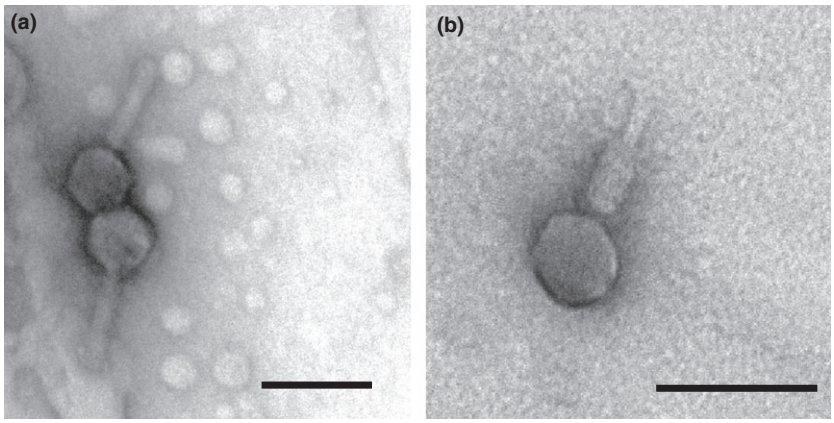

Figure 1 Images from transmission electron microscopy of *Acinetobacter baumannii* phages Bϕ-R1215 (a) and Bϕ-R2315 (b). The bar represents a length of 100 nm.

Ac. baumannii phages Bϕ-R1215 and Bϕ-R2315 at various pHs and temperatures. In the pH stability test of phages, phage Bϕ-R1215 exhibited broad stability of >92% between pH 4–10, the entire range assessed, on day 1, and maintained 64%, 96% and 59% stability at pH 4, pH 7·5 and pH 10 at day 30 respectively (Fig. 2a). Phage Bϕ-R2315 showed broad stability of >80% at pH 4–10 on day 1, and 22%, 92% and 49% stability at pH 4, pH 7·5 and pH 10 at day 30 respectively (Fig. 2b). In the temperature stability test, both phages showed high stability (>96%) at 25°C up to 9 h and maintained at least 30% stability after 9 h at 60°C. However, little or no lytic activities were detected at 70°C for either phage (Fig. 3).

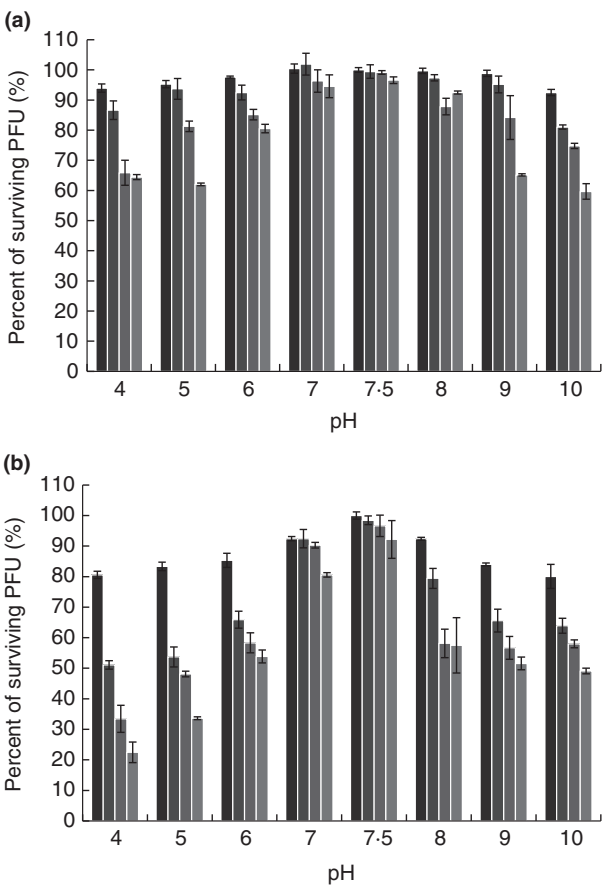

Figure 2 The pH stability of phages Bϕ-R1215 (a) and Bϕ-R2315 (b). Phages were incubated at various pHs (pH 4–10) for 30 days at 4°C, and the results were calculated as the percentage of surviving phages. The error bars denote the standard deviation of three replicates. (■) 1 day; (■) 3 days; (■) 5 days and (■) 30 days.

Host cell lytic activity test

To evaluate the effectiveness and efficiency of phage Bϕ-R1215 and Bϕ-R2315 as antimicrobial agents, we carried out the bacterial lysis test of the phages against host bacteria. The results showed that both phages inhibited the host bacterial growth up to 6 h at all MOIs tested. Interestingly, phage Bϕ-R1215 exhibited lytic activity at an MOI of 0·1 that was as strong as it was at MOI 10 against host bacteria respectively (Fig. 4a,c). On the other hand, inhibition of bacterial growth by phage Bϕ-R2315 was dependent on the MOI (Fig. 4b,d).

Whole genome analysis of *Acinetobacter baumannii* phages

Genome sequencing of *Ac. baumannii* phages Bϕ-R1215 and Bϕ-R2315 generated 708·9 and 466·3 sequence coverage for each genome using 454 junior sequencing. Bϕ-R1215 contained linear double-stranded (ds) DNA containing 44 866 bp and a G+C content of 37·5%. The genome of Bϕ-R2315 contained linear ds DNA with 44 846 bp and a G+C content of 37·6%. Both phages consisted of 85 predicted putative ORFs and there were no putative tRNA genes in either genome. Most of the ORFs were annotated as hypothetical proteins, and only 14 and 12 ORFs were predicted as

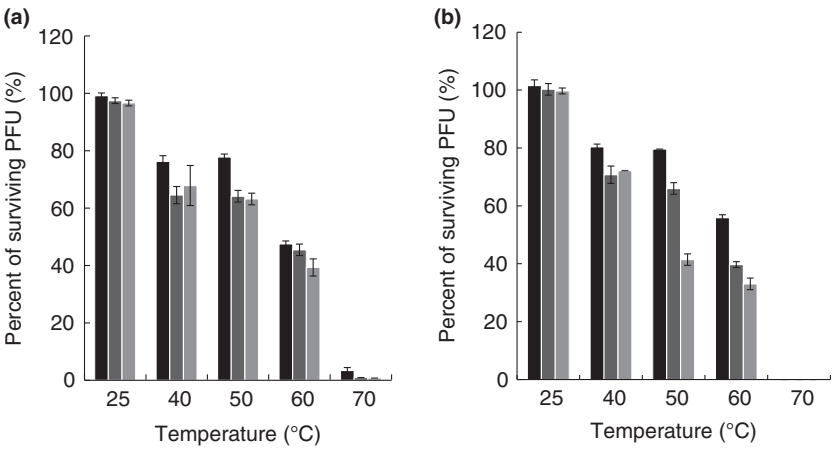

Figure 3 The thermal stability kinetics of *Acinetobacter baumannii* phages Bϕ-R1215 (a) and Bϕ-R2315 (b). The temperature stability of the two phages is represented as a percentage of the surviving phages at 25, 40, 50, 60 and 70°C after 9 h. The error bars denote the standard deviation of three replicates. (■) 3 h; (■) 6 h and (■) 9 h.

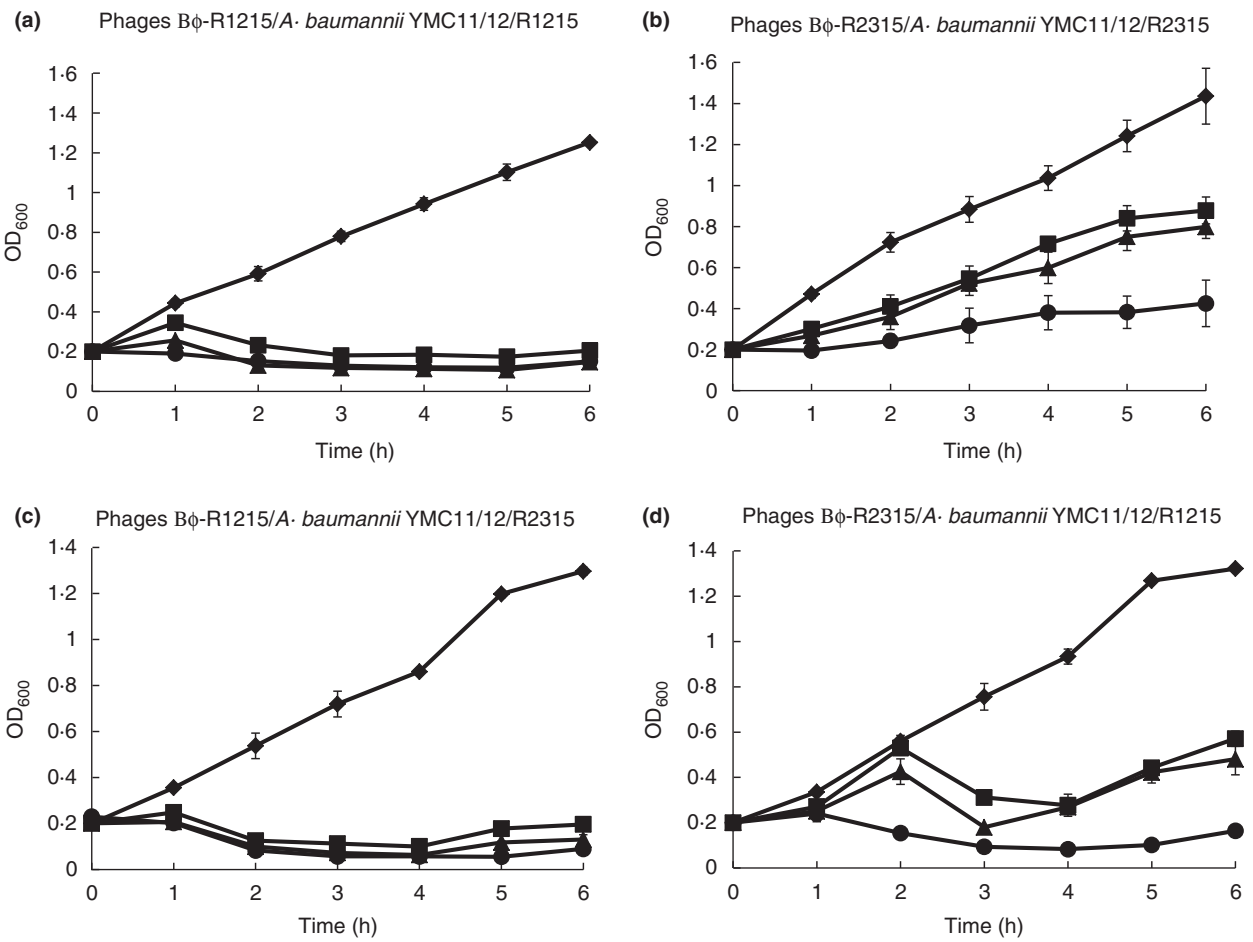

Figure 4 The time course of cell lysis by *Acinetobacter baumannii* phages Bϕ-R1215 (a, c) and Bϕ-R2315 (b, d). Carbapenem-resistant *Ac. baumannii* YMC11/12/R1215 and YMC11/12/R2315 were infected with phages Bϕ-R1215 or Bϕ-R2315 at multiplicity of infection (MOI) 0·1, 1 and 10 respectively. Optical density (OD) was measured at 600 nm. The error bars denote the standard deviation of three replicates. (◆) Control; (■) MOI = 0·1; (▲) MOI = 1 and (●) MOI = 10.

putative functional proteins in the Bϕ-R1215 and Bϕ-R2315 genomes respectively (Fig 5a). All predicted proteins displayed similar amino acid sequences to proteins from *Acinetobacter* phage Bϕ-C62, as assessed using the BLASTp search on the GenBank database (Tables S2 and S3).

Phages Bϕ-R1215 and Bϕ-R2315 shared a high sequence similarity (>90%) with the *Ac. baumannii* phage Bϕ-C62 (Genbank Acc. No. KJ817802) belonging to the Myoviridae family. Also, both the genomes were similar to those of the *Ac. baumannii* phage IME-AB2 (Genbank Acc. No. JX976549) belonging to Myoviridae family (80% similarity) and the *Ac. baumannii* phage AB1 (Genbank Acc. No. HM368260) (57% similarity). The phages also had a very similar genome size of approx. 43–45 kb (Fig. 5b).

The functional ORFs of phages Bϕ-R1215 and Bϕ-R2315 were classified as phage morphogenesis and structural modules, DNA replication and metabolism modules, and lysis modules (Fig. 5a). The detailed annotation information for two phage genomes was listed in Tables S2 and

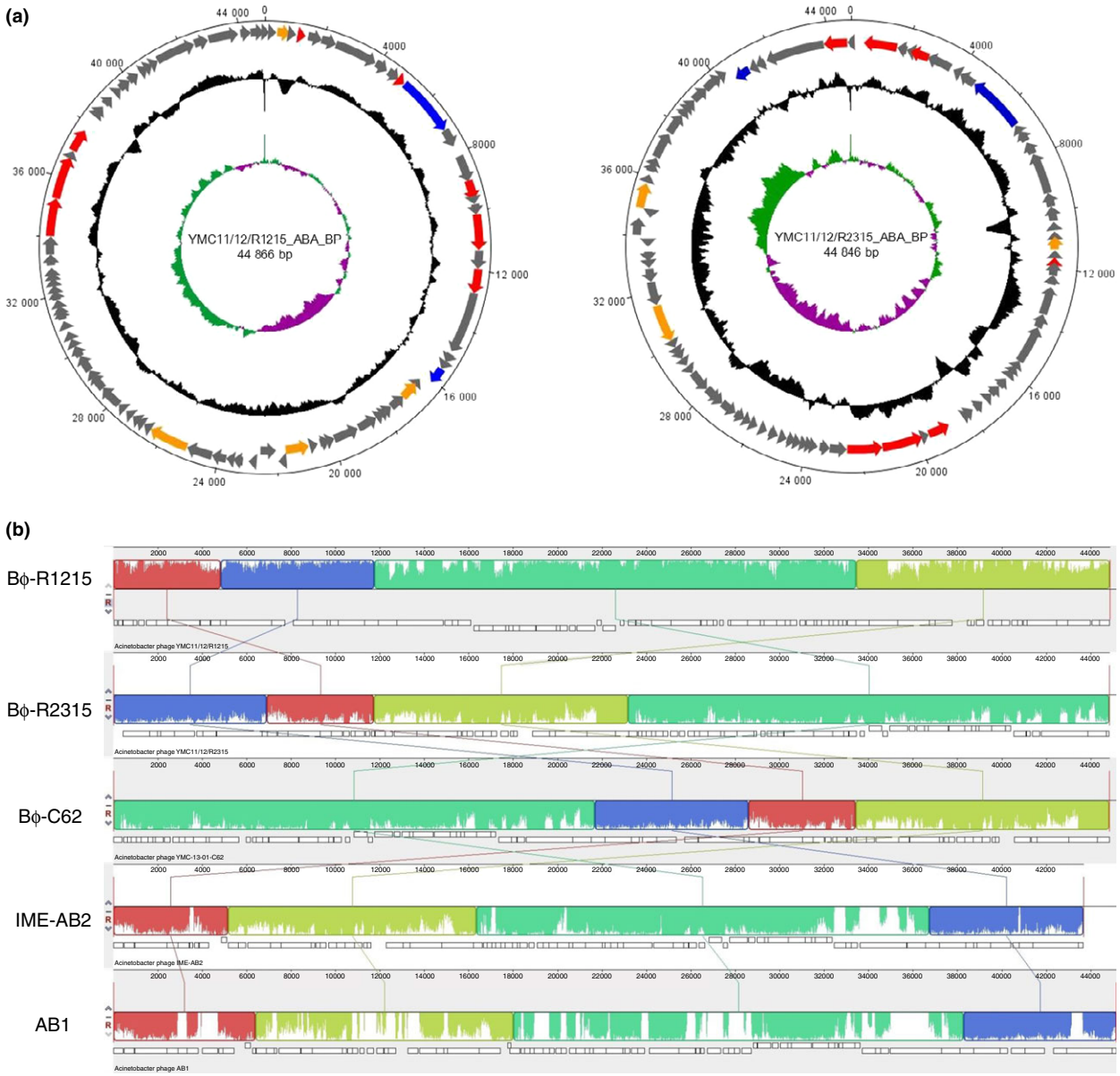

Figure 5 A circular map of the *Acinetobacter baumannii* phages Bϕ–R1215 and Bϕ–R2315 genomes prepared using DNAPlotter (a) and a genome comparison of *Ac. baumannii* phage Bϕ–R1215, phage Bϕ–R2315 and other *Ac. baumannii* phages (Bϕ–C62, IME-AB2, AB1) are represented by MAUVE software at the nucleotide level (b). A genome map of the two *Ac. baumannii* phages illustrates putative open reading frames (ORFs) along with the direction of transcription represented by arrows. Functional proteins, GC content and GC skew are depicted by different colours (a). The connecting coloured lines show the similar DNA regions between predicted ORFs of phage genomes and white bars indicate annotated genes and reverse strands shifted downward (b). Details of annotated genes of the phage Bϕ–R1215 and phage Bϕ–R2315 genomes are listed in the supplemental material (Tables S2 and S3). (■) Morphogenesis protein; (■) DNA replication and metabolism protein; (■) Lysis protein; (■) Hypothetical protein; (■) GC content; (■) GC skew+ and (■) GC skew−.

S3. Most of the contents of the phage proteins associated with morphogenesis, replication, and lysis were similar between phage Bϕ-R1215 and Bϕ-R2315 genomes with regard to protein sequence identity, but the ORF organization and direction were different (Fig. 5b).

Discussion

Recently, *Ac. baumannii* strains have become problematic nosocomial pathogens due to their resistance to most available antibiotics (Perez *et al.* 2007). The phages have been proposed as alternative agents to protect and treat the infectious diseases caused by antibiotic-resistant pathogens. (Tiwari *et al.* 2011; Saussereau and Debarbieux 2012; Wittebole *et al.* 2014). The aim of this study was to isolate and characterize phages that target CRAB and evaluate their effectiveness as an antimicrobial agent to fight these bacteria.

Herein, we collected 45 strains of *Ac. baumannii* that were confirmed as carbapenem-resistant. All the 45 strains were multidrug-resistant *Ac. baumannii* and were resistant to most of the antibiotics (including imipenem and meropenem) used in the study (data not shown).

Until recently, approx. 20 *Acinetobacter* phages have been isolated. Among them, only five *Ac. baumannii* phages—phages IME-AB2 (Peng *et al.* 2014), Acibe1004 (Merabishvili *et al.* 2014), AP22 (Popova *et al.* 2012), ZZ1 (Jin *et al.* 2012) and Abp53 (Lee *et al.* 2011)—belong to the family Myoviridae and have been characterized and/or sequenced. In this study, we isolated and purified two novel *Myoviral Ac. baumannii* phages, Bϕ-R1215 and Bϕ-R2315, infecting CRAB clinical isolates from the sewage water at the hospital. These phages were characterized and completely sequenced.

The host spectrum of the phages is one of the most important features to be considered for further therapeutic application. In this study, a total of 70 bacteria isolated from patients, including both Gram-negative and Gram-positive strains, were used in host spectrum studies; lysis efficacy of the phages was recorded as EOP using the double-layer agar plate method. Overall, *Ac. baumannii* phages Bϕ-R1215 and Bϕ-R2315 showed broad host ranges (approx. 46%) on CRAB isolates. Interestingly, although both phages exhibited a difference of level of EOP on each bacteria strain, they had the same host range on tested bacteria isolates including one of the carbapenem-sensitive *Ac. baumannii* and one of the *Staph. aureus* strains, and they showed relatively high EOPs on the lysed bacteria strain (Table 1).

The adsorption rate and one-step growth curve are the parameters for determining the efficacy of infective phages on host bacteria and their life cycle (Hyman and Abedon 2009). Phage Bϕ-R1215 and Bϕ-R2315 exhibited a rapid adsorption rate of >98% in 5 min (Fig. S1). A one-step growth of these phages showed a latent period of approx. 30 and 40 min, respectively, and a burst size of approx. 43 and 78 PFU per infected cell, respectively, on host bacteria at 60 min (Fig. S2). Phages Bϕ-R1215 and Bϕ-R2315 exhibited similar adsorption rates when compared with *Ac. baumannii* phage AP22 (>99%, 5 min) belonging to Myoviridae. However, phages Bϕ-R1215 and Bϕ-R2315 showed a relatively longer latent period and smaller burst size than previously published data from *Ac. baumannii* phages Abp53 (150 PFU cell^{-1}), ZZ1 (200 PFU cell^{-1}) and AP22 (240 PFU cell^{-1}), also belonging to Myoviridae.

The pH and temperature stability of the phages is considered to be an important factor for optimum activity of the phage on host bacteria. The results suggest that phage Bϕ-R1215 was relatively more stable than phage Bϕ-R2315, that is, phage Bϕ-R1215 maintained higher activity at acidic pH ranges and high temperatures between 50 and 60°C for 9 h (Figs 2 and 3). In comparison with other known *Ac. baumannii* phages, phage Bϕ-R1215 and Bϕ-R2315 exhibited significantly higher stability at a broader pH range than *Ac. baumannii* phage ZZ1, belonging to Myoviridae. Our phages maintained significant stability at high acidic (pH 4) and alkaline (pH 10) pHs up to 30 days (Fig. 3). *Acinetobacter baumannii* phages ZZ1 and AB1 (Yang *et al.* 2010), belonging to Siphoviridae, have shown high temperature stability during 1 h incubation at 50–60°C, and phage Bϕ-R1215 and Bϕ-R2315 showed relatively high stability up to 9 h between the same temperature range (Fig. 3). These results suggest that our novel phages are at least as stable in the various clinical environments as *Ac. baumannii* phage ZZ1 and phage AB1.

To assess the phages as a potential antimicrobial agent, we performed the host cell lysis test against host bacteria *in vitro*. OD_{600} of bacterial growth infected with phage Bϕ-R1215 at all different MOIs did not increase up to 6 h. Phage Bϕ-R2315 also inhibited host bacterial growth (i.e. OD_{600}) dose-dependently and showed the highest lysis activity at an MOI of 10 (Fig. 4). Both *Ac. baumannii* phage Bϕ-R1215 and Bϕ-R2315 exhibited significant inhibition efficacy of bacterial growth in LB culture.

As shown in Fig. 5, despite the high level of DNA sequence similarity between phage Bϕ-R1215 and Bϕ-R2315 genomes, there is a substantial divergence in gene organization. The sequence similarity may be associated with the similar morphologies and the same host ranges. Also, the genes associated with lysogeny, such as repressor and integrase genes, or virulence factors (Plunkett *et al.* 1999) were not identified in either of phage genomes (Tables S2 and S3). Therefore, based on the *in vitro* and *in silico* results, we determined that phage Bϕ-R1215 and Bϕ-R2315 are lytic bacteriophages.

By comparing the genome of other phages, phages Bϕ-R1215 and Bϕ-R2315 genomes exhibited a high level of DNA sequence similarity and similar genome size with *Ac. baumannii* phage Bϕ-C62 (Myoviridae), IME-AB2 (Myoviridae) and AB1 (Siphoviridae), however, overall genome organization between these phages differs. Phages IME-AB2 and AB1 have a circular genome, while phages Bϕ-R1215 and Bϕ-R2315 have a linear genome, and it was confirmed as the PCR by using primers designed (data not shown). The detailed genome analysis of the phages provides us not only crucial molecular insight into the differences between phages but also expansion of our understanding of the genetic diversity within *Acinetobacter* phage genomics. Indeed, these analyses are essential process as significant parameters to assess the safety of the phages prior to their use as therapeutic agents in the future.

Additionally, to visualize the phage proteins, purified each phage sample was separated by on 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The proteomic patterns also were similar between phage Bϕ-R1215 and Bϕ-R2315, containing three major bands (approx. 30, 16, and 10 kDa) (Fig. S3).

Here, we report in detail the characterization and complete genome sequence of two novel *Myoviral Ac. baumannii* phages Bϕ-R1215 and Bϕ-R2315 infecting CRAB clinical strains. The *in vitro* characterization *in silico* analysis results obviously shows that novel *Ac. baumannii* phages Bϕ-R1215 and Bϕ-R2315 have potential as antimicrobial alternatives to control CRAB strains becoming a serious threat to the hospital settings.

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Conflict of Interest

No conflict of interest declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. The adsorption rate of *Ac. baumannii* phages Bϕ-R1215 (a) and Bϕ-R2315 (b).

Figure S2. One-step growth curve of *Acinetobacter* phages Bϕ-R1215 (a) and Bϕ-R2315 (b).

Figure S3. The profiles of phage structural proteins by silver-stained SDS-PAGE. Lane 1: Bϕ-R1215; lane 2: Bϕ-R2315; lane 3: Molecular weight marker.

Table S1. Host bacteria strains, *Ac. baumannii* YMC11/12/R1215 and YMC11/12/R2315, used in this study and their antibiotic susceptibility results[a].

Table S2. *Ac. baumannii* phage Bϕ-R1215 ORFs summary.

Table S3. *Ac. baumannii* phage Bϕ-R2315 ORFs summary.